



# The concurrent effects of *azurin* and *Mammaglobin-A* genes in inhibition of breast cancer progression and immune system stimulation in cancerous BALB/c mice

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## Abstract

In the present study, the simultaneous application of *azurin* gene of *P. aeruginosa* and *MAM-A* antigen on the induction of immune responses against breast cancer tumors was investigated in BALB/c mice. The pBudCE4.1-*azurin*-*MAM-A* recombinant vector was generated and prepared at a large scale. This recombinant vector alone or combined with chitosan nanoparticles was infused into the hip muscle of animals. Animals were divided into the “prevention” and “therapy” categories. The animals of prevention category were first, immunized by a recombinant vector and then exposed to chemical cancer inducers; while the animals in the therapy category were first treated with chemical compounds and then infused by a recombinant plasmid. The tumor tissues, infusion sites, and blood specimens were collected and examined by serological, molecular, and histological tests. The breast tumor incidence in the infused animals by recombinant plasmid alone or combined with nanoparticles (in both prevention and therapy categories) compared with infused mice by empty pBudCE4.1 vector was significantly decreased ( $p < 0.05$ ). These results were supported by histological studies using H&E staining. The ELISA and q-real-time PCR techniques showed the range of IFN- $\gamma$ , IL-12, IL-4, and IL-17A cytokines in the infused mice by recombinant vector alone or combined with nanoparticles compared to the healthy mice and infused animals by intact pBudCE4.1 were significantly increased ( $p < 0.05$ ). Accordingly, the expression of the tumor markers *CEA*, *Krt20*, and *Muc1* were significantly decreased in treated mice either by the sole recombinant vector or combined with nanoparticles ( $p < 0.05$ ). These findings indicated that pBudCE4.1-*azurin*-*MAM-A* recombinant vector plays an essential role against the formation and expansion of breast tumors in the animal model. In addition, this recombinant vector is safe and has the proper ability to stimulate the immune system. In addition, the chitosan nanoparticle represents a promising adjuvant for DNA vaccine delivery, which improves the immune system stimulation and boosts the vaccine performance.

**Keywords** Azurin · MAM-A · Breast cancer · DNA vaccine · Antitumor activity

## Abbreviations

MAM-A Mammaglobin-A  
*P. aeruginosa* *Pseudomonas aeruginosa*

## Introduction

Breast cancer is one of the most common types of cancers in women worldwide in all age groups. It is a second leading cause of death after lung cancer and one of the three most commonly occurring cancers in the world (Ly et al. 2011; Balekouzou et al. 2016). According to the reports published by the World Health Organization (WHO) in the year 2018, the most common cancers in the world are breast (2.09 million cases), lung (2.09 million cases), colon and rectum (1.8 million cases), prostate (1.28 million cases), and stomach (1.03 million cases) cancers (Aragon et al. 2015; Bray et al. 2018). Familial and genetic histories, increasing age, lifestyle, geographic region, nulliparity, late age at first birth, pregnancy at an early and late ages, late menarche, early menopause, late age of menopause with high intake

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of polyunsaturated fats, and early menstrual periods before age 12 years are more risk factors for breast cancer (Kruk 2007; Anderson et al. 2014; Anjum et al. 2017). Due to the limitations of existing therapies for breast cancer including surgery, radiation therapy, chemotherapy, hormone and biological therapies, endocrine therapy, and as well as the possibility of metastasis, the lack of definitive treatment in advanced disease, and the lack of an appropriate vaccine for prevention and treatment of this type of cancer, it is very important to find a definitive and effective therapeutic method based on molecular methods and recombinant DNA vaccines (Li and Petrovsky 2016; Paluch-Shimon et al. 2016; Lukong 2017). One of the vaccine strategies for the disease that has been reported and studied is the application of DNA plasmid for antigenic protein coding. This recombinant plasmid is infused directly into the muscle of the animal to an expression of protein antigens. In addition, this type of vaccines is subunits and the antigen expression by this DNA plasmid activates both humoral and cellular immune response. The advantages of genetically engineered DNA vaccines are noninfectious, cheapness, safety, easy to grow, store and production, heat resistance, and long-term safety of application (Khan 2013; Zhang and El Zowalaty 2016). Thereupon, gene vaccines lead to the long-term expression of the antigen and beget a memory of the desired immune system (Felberbaum 2015). Nowadays, there is a lot of evidence about the interaction between the immune system and breast cancer, therefore, this type of cancer has become a good target for research on cancer vaccine (Farkona et al. 2016). One of the most important bacteria that its toxins and enzymes have been therapeutic properties is *Pseudomonas aeruginosa* (*P. aeruginosa*). The azurin bacteriocin in this bacterium is a periplasmic copper-containing protein with 14-kDa molecular weight that consists of 128 amino acids (Bernardes et al. 2013; Karpiński and Adamczak 2018). This bacteriocin has an anticancer effect against breast cancer tumors via interaction with p53 and binding to the EphB2 tyrosine kinase (extracellular receptor protein) and can prevent the tumor progression (Bernardes et al. 2010; Fialho et al. 2016; Gao et al. 2017).

Mammaglobin-A (MAM-A) is a 10-kD secretory protein containing 93 amino acids. This protein is encoded by the *SCGB2A2* gene and expressed in most cases of primary and metastatic human breast cancers (Soysal et al. 2014). MAM-A through the eliciting of MAM-A-specific CD8 T cell in a body and due to the unique expression in breast cancer cells is known as a very specific molecular marker in breast cancer patients. In addition, this protein is an appropriate target for immunosuppressive therapy in these patients (Kim et al. 2016). Therefore, in this study, the encoding genes of azurin bacteriocin of *P. aeruginosa* and human MAM-A were used in the prepared recombinant pBudCE4.1-azurin-MAM-A gene construct and their

simultaneous effects on incitement of the immune system and breast cancer tumors in cancerous BALB/c mice were evaluated.

## Materials and methods

### pBudCE4.1-azurin-MAM-A construct preparation

In this research, the recombinant pBudCE4.1-azurin-MAM-A was designed and ordered to the Genaray Biotech Co., Ltd. (Shanghai, China) for synthesis. In addition, the empty pBudCE4.1 vector was obtained from this company. For the expression of this recombinant vector in the eukaryotic host, the codon optimization of the *azurin* and *MAM-A* (target genes) sequences for better expression was done by Genaray Biotech company. DNA sequencing and restriction enzymatic digestions by *Pst*I, *Kpn*I, *Xba*I, and *Xho*I were done for reliability and validity of artificial gene synthesis and pBudCE4.1-azurin-MAM-A recombinant vector generation.

The pBudCE4.1 vector possesses two different multiple cloning sites (MCSs) that in this study both of *azurin* and *MAM-A* genes were cloned independently into these separate MCSs. The pBudCE4.1 (empty vector) has a length of 4.6 kb and capable to express in human and animal hosts which has two eukaryotic promoters (human cytomegalovirus (CMV) and human elongation factor 1 alpha (EF-1 alpha) promoters). Both *azurin* and *MAM-A* genes with the length of 1287 and 1309 bp, respectively, were inserted in the pBudCE4.1-azurin-MAM-A recombinant vector near these strong promoters to simultaneous expression of both genes.

### Bacterial transformation and plasmid extraction

The *E. coli* *NovaBlue* strain (Novagen) was overnight cultured in Luria–Bertani (LB) broth (Sigma, St. Louis, Mo. USA) by incubation at 37 °C with shaking at 180 rpm. The *NovaBlue* competent cells were prepared using calcium chloride (CaCl<sub>2</sub>) and a brief (90 s) heat shock (42 °C) treatments. These cells were used for separate transformation of pBudCE4.1-azurin-MAM-A and empty pBudCE4.1 vectors. Then, transfected bacteria were cultured in low salt LB agar medium in the presence of 25 µg/mL of zeocin antibiotic for colony screening. The transformation accuracy was first confirmed by colony-polymerase chain reaction (PCR) screening using specific primers and then positive colonies were cultured into the 5 mL of LB broth containing zeocin. Finally, the mini-prep plasmid DNA extraction from cultured bacterial cells was performed using YTA Miniprep Kit according to the recommendations of the manufacturer (Yekta Tajhiz Azma Co, Tehran, Iran). Determining the yield and quality of purified plasmids were measured in a ratio

of absorbance at 260 nm and 280 nm, using a NanoDrop spectrophotometer (Thermo Scientific™ NanoDrop 2000, Wilmington, DE, USA) according to the method described by Sambrook and Russell 2001. The presence of target genes (*azurin* and *MAM-A*) in the isolated recombinant vector was investigated by PCR and digestion with several restriction enzymes (*Nco*I, *Kpn*I, *Apa*I, *Sma*I, and *Sac*I). Each amplified product and digested fragments were loaded on a 2% agarose gel in the presence of a 1 kb DNA marker (CinnaGen, Iran) and were electrophoresed for 45 min at 80 V in running buffer (TBE 1%). The gel was visualized under UV light using the UVIDoc gel documentation system (Uvitec, UK) after an appropriate ethidium bromide (2 µg/mL) staining.

### Conventional PCR of target genes

The sequences of target genes, including *azurin* and *MAM-A* were obtained from the National Center for Biotechnology Information (NCBI) GenBank sequence database. The PCR oligonucleotide primers for each gene were designed with the assistance of the computer program Oligo 4.0 (National Biosciences, Inc., Plymouth, MN) (Table 1). The sequence of each primer was compared to a query sequence of GenBank data using BLAST (Basic Local Alignment Search Tool) and Multiple Sequence Alignment by CLUSTALW (Kyoto University Bioinformatics Center; <http://www.genome.jp/tools/clustalw>). The PCR amplification was performed

in a final volume of 25 µL containing 1 µL of 0.2 µM dNTP mix, 2 µL of MgCl<sub>2</sub> (2 mM), 2 µL 1× PCR Buffer (all CinnaGen, Tehran, Iran), 1 unit (0.2 µL) of CinnaGen *Taq* DNA polymerase, 1 µL (1 µM) of each forward and reverse primer, and 2 µL (50 ng) of pBudCE4.1-*azurin*-*MAM-A* recombinant vector. A negative control contains all reaction components, except the template plasmid DNA. Both target genes were amplified in a thermal cycler (Gene Atlas G; ASTEC Co., Seoul, Korea). The following thermal cycling program was used to obtain products: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 65 °C and 66 °C for *azurin* and *MAM-A* genes, respectively, elongation at 72 °C for 50 s, and a final elongation at 72 °C for 10 min. The PCR products were electrophoresed through 1% agarose gel and stained with ethidium bromide and finally visualized under UV light according to the procedure used previously.

### Large-scale preparation of the vectors

After transformation confirmation, the transformed bacterial cells were cultured in 250 mL LB broth containing zeocin antibiotic. Then, the bacterial culture was deposited and the recombinant pBudCE4.1-*azurin*-*MAM-A* plasmids were extracted in a large volume using a YTA Maxiprep Kit (Yekta Tajhiz Azma Co, Tehran, Iran) according to the manufacturer's guidelines for the vector infusions in BALB/c

**Table 1** The details of oligonucleotide primers that were used for RT-PCR and q-real-time PCR

Gene	Primers name	Sequences	Annealing temperature (°C)	Product length (bp)	Accession number
<i>Azurin</i>	Azu-F	5'-ATGCTACGTAAACTCGCTGCC-3'	65	292	M30389
	Azu-R	5'-TGTCGTCGGGCTTCAGGTAATC-3'			
<i>MAM-A</i>	Mam-F	5'-CAGCGGCTTCCTTGATCCTTG-3'	65	221	NM_002411
	Mam-R	5'-TGGCATTGTCGTCTATGAACCTCTG-3'			
<i>IFN-γ</i>	IFN-γ-F	5'-GCCTAGCTCTGAGACAATGAACG-3'	64	188	M28621
	IFN-γ-R	5'-GCCAGTTCCTCCAGATATCCAAG-3'			
<i>IL-4</i>	IL-4-F	5'-TCACAGGAGAAGGGACGCCATG-3'	66	246	NM_021283
	IL-4-R	5'-TGGACTTGGACTCATTCATGGTGC-3'			
<i>IL-12</i>	IL-12-F	5'-TGACACGCCTGAAGAAGATGAC-3'	64	325	M86671
	IL-12-R	5'-ACTGCTACTGCTCTTGATGTTGAAC-3'			
<i>IL-17A</i>	IL-17-F	5'-CTACAGTGAAGGCAGCAGCGATC-3'	66	263	NM_010552
	IL-17-R	5'-CTTTCCCTCCGCATTGACACAG-3'			
<i>CEA</i>	CEA-F	5'-GGCACTAATAAGACTACAACAGGGC-3'	64	223	X53084
	CEA-R	5'-GTTCTTTGACTGTGGTGTGGTGAC-3'			
<i>KRT20</i>	KRT20-F	5'-ATTGAGGTTCAAGTCACGGAGC-3'	66	206	NM_023256
	KRT20-R	5'-TCTGGCGTTCTGTGTCACTCCTG-3'			
<i>Muc1</i>	Muc1-F	5'-CCACCAGTTCTCCAGTCCACAGTAG-3'	65	233	NM_013605
	Muc1-R	5'-CGTCACTTTGGTAGTAGAGAATGGC-3'			
<i>GAPDH</i>	GAPDH-F	5'-TCCCGTAGACAAAATGGTGAAGG-3'	65	261	XM_017321385
	GAPDH-R	5'-ATGTTAGTGGGGTCTCGCTCCTG-3'			

mice. The optical density (OD) of purified plasmids was measured at a wavelength of 260/280 nm as described above.

### Preparation and in vitro characterization of chitosan nanoparticles

Chitosan nanoparticles were synthesized via the ionic gelation method and sodium tri-polyphosphate (TPP) anions. Based on this method, chitosan nanoparticles (Sigma-Aldrich, USA) with low molecular weight and moderate were dissolved in 1% acetic acid aqueous solution at (2 mg/mL) under the magnetic stirring at 800 rpm for 24 h incubation at room temperature. The pH of this solution was adjusted to 5 using 0.5 M NaOH. Then, the chitosan solution was filtered by 0.45- $\mu$ m Millipore filter to remove any unsolved chitosan. In addition, TPP solution was prepared at a concentration of 0.7 mg/mL by dissolving in enough deionized (DI) water. The pH of the solution was adjusted to 4 with acetic acid and then was passed through a 0.2  $\mu$ m Millipore filter. To prepare the chitosan nanoparticles, the TPP solution was slowly dropped to 50 mL of an acetic acid solution containing chitosan. Then, this solution was shaken on a magnetic stirrer at 800 rpm in room temperature for 45 min. Finally, the prepared nanoparticles suspension were centrifuged at 14,000 rpm (4 °C) for 15 min and were powdered and freeze-dried using a Virtis Advantage Plus

freeze-dryer (SP Scientific, Warminster, USA) and stored at 4 °C. The size, surface charge, zeta potential, and Polydispersity Index (PDI) of the chitosan nanoparticles were determined using a Malvern Nano ZS90 Zetasizer Nanoseries system (Malvern Instruments, Malvern, UK). The weights of freeze-dried nanoparticles were also measured and the morphological analysis of nanoparticles was investigated by scanning electron microscope (SEM). One drop of lyophilized chitosan nanoparticles was coated with a gold-plated metal and nanoparticle surface properties and their shape were observed using SEM. For animal infusions, an equal volume of both chitosan nanoparticles solution (1%) and the plasmid DNA solution (2000  $\mu$ g/mL dissolved in PBS) were mixed well by pipetting and were incubated at 55 °C for 1 h.

### Ethics statement and animal grouping

This study was approved by the Research Ethics Committees of the Deputy of research and technology of Islamic Azad University of Shahrekord Branch, Shahrekord, Iran on October, 10th 2017 with ethics code: IR.IAU.SHK.REC.1397.049. The infusions were done in 78 female BALB/c mice with 5 weeks age and 18 g average weight. These animals were divided into six groups according to Table 2. In all treatments, six mice were considered normal control (healthy mice). For infusions, 1000  $\mu$ g/mL of both

**Table 2** The animal groups and schedule of the treatments of BALB/c mice in this study

Group	Number of mice	Explanation
<b>Prevention</b>		
A	12	Infusion by recombinant vector <sup>a</sup> (3 times, days 0, 7, and 15) and 15 days after the last injection gavage by DMBA <sup>b</sup> (4 times, days 30, 51, 72, and 93) and 1 month later (day 123) infusion by MNU <sup>b</sup> (single dose), finally follow-up within 3 months for breast tumor formation
B	12	Infusion by recombinant vector plus chitosan nanoparticles (3 times, days 0, 7, and 15), and 15 days after the last injection gavage by DMBA (4 times, days 30, 51, 72, and 93) and 1 month later (day 123) infusion by MNU (single dose), finally follow-up within 3 months for breast tumor formation
C	12	Infusion by only pBudCE4.1 vector without target genes (pBudCE4.1) for 3 times (days 0, 7, and 15) and 15 days after the last injection gavage by DMBA (4 times, days 30, 51, 72, and 93) and 1 month later (day 123) infusion by MNU (single dose), finally follow-up within 3 months for breast tumor formation
<b>Therapy</b>		
D	12	Gavage by DMBA (4 times, days 0, 21, 42, and 63) and 1 month later (day 94) infusion by MNU (single dose) and after 3 months (follow-up for breast tumor formation) infusion by a recombinant vector (3 times, days 184, 191, and 198)
E	12	Gavage by DMBA (4 times, days 0, 21, 42, and 63) and 1 month later (day 94) infusion by MNU (single dose), and 3 months later (follow-up for breast tumor formation) infusion by recombinant vector plus chitosan nanoparticles (3 times, days 184, 191, and 198)
F	12	Gavage by DMBA (4 times, days 0, 21, 42, and 63) and 1 month later (day 94) infusion by MNU (single dose) and after 3 months (follow-up for breast tumor formation) infusion by the only pBudCE4.1 vector (3 times, days 184, 191, and 198)
<b>Healthy mice</b>		
G	6	Normal or healthy mice (without infusion)

<sup>a</sup>Recombinant vector: pBudCE4.1-azurin-MAM-A

<sup>b</sup>DMBA and MNU: chemical compounds as cancer inducers

pBudCE4.1 (empty vector) and pBudCE4.1-azurin-MAM-A plasmids was separately dissolved in PBS. In addition, a 2% of chitosan nanoparticles solution was prepared in PBS and dissolved in 1000 µg/mL of the recombinant vector that dissolved in PBS (half as much as half). For each BALB/c mice in all groups, 100 µL of these DNA solutions (as a DNA vaccine) was infused into the quadriceps muscle of animals of each group [in the form of intramuscular (IM) injection] according to the schedule of injections in Table 2. In this study, due to the synergistic effects of the 7,12-dimethylbenz[a]anthracene (DMBA) and 1-methyl-1-nitrosourea (MNU) in mammary carcinogenesis, these chemical compounds were used for induction of breast cancer in both prevention and therapy categories of female BALB/c mice. DMBA was dissolved in 100 µL of corn oil with a dose of 1 mg/week for gavage of each mouse until completing the total dose of 1, 3, 6 or 9 mg (Oliveira et al. 2015). One month after the last DMBA gavage, a single dose of MNU (50 mg/kg or 1 mg for each BALB/c mice) dissolved in corn oil was intraperitoneally (IP) injected to induce the breast cancer tumors (Roomi et al. 2005; Boshra and Hussein 2016). All groups after the last treatment were followed for 3 months to investigate the breast cancer tumor development. At this time, in different intervals, one mouse in the control groups (C and F groups that infused by only pBudCE4.1 and exposed to the chemical compounds) was killed and examined for breast tumor formation to tumor size investigation and histological studies. All mice were weighed weekly and were examined until death. Finally, after follow-up for breast tumor formation, the mice of all groups [the infused mice by recombinant vector alone or combined with chitosan nanoparticles, and as well as intact plasmid (pBudCE4.1) as a control group] were killed. Then, the complete blood sample of each mouse was taken directly by needle aspiration of a heart and was separately collected in ethylenediaminetetraacetic acid (EDTA) tubes and normal 1.5-mL micro-tube (clot blood) for molecular and serological studies, respectively, in further tests. Afterward, the mice of each group were autopsied and the tumor texture was analyzed and compared for tumor size and histological analysis. In addition, the muscle of the injection site (mouse thigh) was removed and stored at  $-70^{\circ}\text{C}$  for investigation the expression of target genes (*azurin* and *MAM-A*) of the recombinant vector. One part of the breast tumor tissue was separately obtained for RNA extraction and one slice was gathered in 10% formalin for histological studies.

### RNA extraction and cDNA preparation

Total RNA was isolated from collected blood and homogenized tissue (injection site, tumor tissue, and normal tissue) samples of all group mice. RNA purification was carried out by 1 mL ice-cold RNX plus (RNX plus™ Kit Cinnagen,

Tehran, Iran) according to the manufacturer's instructions. The concentration and quality of extracted RNA was quantified using NanoDrop spectrophotometer. Each cDNA sample was synthesized from 2 µg of the extracted total RNA using both oligo dT and random hexamer mix (50 µM) according to the standard protocol of cDNA synthesis kit (Yekta Tajhiz Azma, Tehran, Iran).

### Gene expression

The expression of target genes of pBudCE4.1-azurin-MAM-A recombinant vector (*azurin* and *MAM-A* genes) in the mice injection sites were evaluated by reverse transcription PCR (RT-PCR). The RT-PCR procedure and thermal conditions were the same as mentioned above except template DNA that was replaced by cDNA sample and the products were analyzed on a 1% agarose gel.

The q-real-time PCR with SYBR green detection was used for the survey of important cytokines [including interferon- $\gamma$  (*IFN- $\gamma$* ), *IL-4*, *IL-12*, and *IL-17A*] in the blood samples and tumor marker [*Muc1* or *Mucin 1*, *KRT20* (encodes cytokeratin 20 protein), and carcinoembryonic antigen or *CEA*] expression level in the breast tumor tissues. As mentioned before, the expression of these tumor markers increases in the primary and advanced breast cancer, and they are useful and ideal molecular markers for monitoring of breast cancer tumors (Lacroix 2006; Kufe 2013; Lasa et al. 2013). The sequence of each gene was obtained from the GenBank sequence database of NCBI and all PCR primers were designed using Gene Runner version 3.05 and Primer Premier 5.0 software and their queries were analyzed by BLAST. The list of gene name and the primer sequence used for real-time PCR is shown in Table 1. In all q-real-time PCR reactions, a *GAPDH* reference gene was used as an internal control for comparison and normalization of the gene expression level. A Corbett Rotor-Gene 6000 real-time rotary analyzer (Corbett, Australia) was used for amplification and melting curve analysis. A serial dilution of the cDNA sample at a concentration of 1:5, 1:25, 1:125, 1:625, and 1:3125 were prepared and standard curve analysis of each primer set was done. The cDNA sample at a concentration of 1:5 was suitable for q-real-time PCR amplification. All cDNA samples using the standard curve method were evaluated and the q-real-time PCR was performed in triplicate for each gene. The reaction mixture in a 0.2-mL micro-tube at a final volume of 13 µL consisted of 6.5 µL of YTA 1× SYBR Green qPCR Mix (Yekta Tajhiz Azma, Tehran, Iran), 0.5 µL of each forward and reverse primer (2 µM), 1 µL cDNA specimen (50 ng), and 4.5 µL of distilled water. The q-real-time PCR was performed under the following temperature conditions: pre-denaturation at  $95^{\circ}\text{C}$  for 3 min, 40–45 cycles of denaturation at  $94^{\circ}\text{C}$  for 30 s, annealing (temperatures are according to Table 1 for each



gene) for 3 min, and elongation at 72 °C for 30 s followed by melting curve analysis at a temperature of 72 °C to 95 °C (1 °C/s). The relative expressions were monitored by Rotor-Gene Real-time analysis software version 6.0 (Qiagen, Inc., Valencia, CA, USA) using the comparative C<sub>ts</sub> ( $2^{-\Delta\Delta C_t}$ ) of the target and reference genes (Livak method).

### Sandwich ELISA test

The enzyme-linked immunosorbent assay (ELISA) test was done for evaluation of the serum level of IFN- $\gamma$ , IL-4, IL-12 (IL-12/P40), and IL-17A cytokines in all collected mice serum samples according to the method described by each ELISA kit (Hangzhou Eastbiopharm Co., Ltd., Hangzhou, China with Cat. No: CK-E11382 for IFN- $\gamma$ ; Cat. No: CK-E20011 for IL-4; Cat. No: CK-E90740 for IL-12, and Cat. No: CK-E92107 for IL-17A). Standard dilutions were performed at concentrations of 640 ng/L, 320 ng/L, 160 ng/L, 80 ng/L, and 40 ng/L using original standard and standard diluents. All ELISA kits were based on the principle of double-antibody sandwich technique and 40  $\mu$ L of serum of each BALB/c mice was added to the each test wells. Fifteen minutes after adding the stop solution, the optical density (OD) of all wells of an ELISA plate was measured under 450 nm wavelength by Stat Fax-2100 ELISA plate reader (Awareness Technology, Palm City, FL). The standard curve linear was calculated and drawn according to the standards' concentration and the corresponding OD values.

### Tissue examination

In this study, after the gavage of DMBA and MNU injection, during and 3 months after the last treatment, all mice in each group were tested for breast cancer tumors and histological studies. Mice in each group were weighed daily and the incidence and development of breast cancer tumors were examined weekly. The diameter of the tumor was measured with the Vernier caliper and the tumor volume was calculated according to the formula:  $V = 0.5a \times b^2$  ( $a$ : is the longest diameter of the tumor,  $b$ : is the shortest diameter of the tumor). At the end of the treatment period, the animals were anesthetized with diethyl ether. In cancerous mice, the area around the tumor was shaved and splitting. Afterward, the tumors were completely removed in a sterile condition and one part of the tumor tissue was placed in 10% formalin and one slice was separately used for a molecular test.

### Preparation of the tissue sections by microtome

Tumor tissues were processed using an automatic tissue processor (Leica Histokinette 2000; Leica Microsystems, Wetzlar, Germany). All steps were carried out in accordance with

the manufacturer's specifications and instructions with minor modifications which are summarized below. After dipping the tissues with increasing degrees of alcohol, the clarity of the samples from alcohol was done using a replacement with xylene and then with paraffin embedding. Then, the samples by maintaining their orientation were molded in paraffin. Two pieces of molds were placed on a flat glass or stone surface and molten paraffin was poured into the molds. All samples were placed with paraffin in the form of preservation and the specification of each section was recorded and bonded to paraffin in the block. After cooling and tightening of the paraffin, the molds were separated from the solid paraffin containing the sample by a brief blow. The molds were kept in the refrigerator until they were cut with a microtome. The cutting of paraffin blocks containing the sample was prepared by a microtome with a thickness of 1  $\mu$ m. To remove of wrinkles, the samples were transferred to the slide (that previously placed on the albumin glue) at a 30° slope on a water bath at 45 °C. The slides were transferred to the heater and then placed on a wooden board and were placed at 39 °C for 12 h until the samples completely stuck to the slide. One slide from each block was subjected for haematoxylin and eosin (H&E) staining for histopathological studies.

### H&E staining of the tissue sections

The H&E or HE stain was used for staining of the tissue sections. Before staining, the slides were placed in the oven for 1–2 h at 55 °C for deparaffinization. After removing the xylol and alcohol of the cells, a haematoxylin (Harris) solution was used for 20–40 min to stain of the tissue sections. Then, the tissue sections were washed in tap water for 1–5 min, until sections turn blue. The sections were differentiated in 70% ethanol containing 1% HCl for 5 s and were washed for 1–5 min, in tap water until turning blue. Next, the sections were stained by Eosin solution for 10 min and were washed again for 1 to 5 min in tap water and let to dehydrate, clear, and mount. To permanently seal the samples, the Canada balsam glue was used between slide and lamel and thereafter the slides were air-dried for 12 h to completely dry.

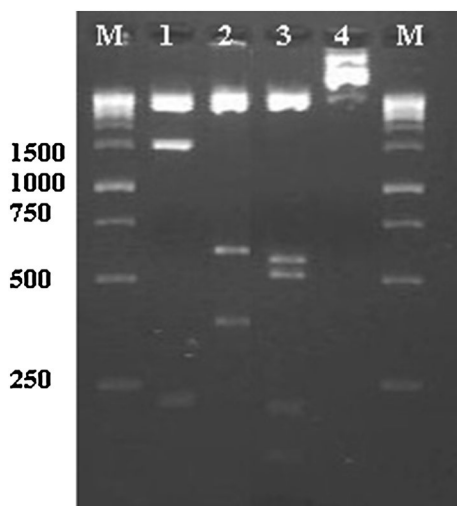
### Statistical analysis

All experiments were run in triplicate and repeated at least three times. All data were entered into the Social Sciences software (SPSS, Inc., Chicago, IL, USA) version 20 and the mean difference between groups were calculated by independent  $T$  test or analysis of variance (ANOVA) statistical methods. The graphs were prepared using GraphPad Prism version 5.01 for Windows (GraphPad Software, San Diego, CA, USA, <http://www.graphpad.com>). A  $p < 0.05$  was considered statistically significant.

## Results

### Confirmation of transformation

The presence of target genes, including *azurin* and *MAM-A* in pBudCE4.1-*azurin*-*MAM-A* recombinant vector, was confirmed by Genaray Biotech company using enzymatic digestions and DNA sequencing. The transformation



**Fig. 1** The fragments of digested pBudCE4.1-*azurin*-*MAM-A* recombinant vector using restriction enzymes on 2% agarose gel electrophoresis (M: 1-kb DNA molecular marker (Thermo Fisher Scientific), line 1: digested recombinant vector by *Nco*I: 220 bp, 1471 bp (containing *azurin* gene), and 3657 bp, line 2: restricted plasmid by *Kpn*I and *Apa*I including 590 bp, 19 bp, 368 bp (containing *MAM-A* gene), and 4371 bp, line 3: restriction fragments by *Sma*I and *Sac*I: 187 bp, 551 bp, 132 bp, 492 bp (containing *azurin* gene), and 3986 bp, and line 4: undigested recombinant plasmid (5348 bp), respectively)

accuracy of the recombinant vector into *E. coli* was verified using enzymatic digestions and indicated the successful transformation of the vector into the host for increasing the amount of plasmid (Fig. 1).

### Physicochemical characterization of chitosan nanoparticles for plasmid DNA delivery

In this study, the chitosan nanoparticles were used for better uptake of plasmid DNA. The particle size, zeta potential, molecular weight, particle charge, and a dispersion index of chitosan nanoparticles were evaluated using dynamic light scattering (DLS) instrument and Zetasizer nano ZS ZEN3600 (Malvern Malvern Zetasizer Nano ZS, ZEN 3600) at a wavelength of 633 nm in 25 °C. The zeta potential analysis of chitosan nanoparticles showed that these nanoparticles successfully prepared and 98.8% of these particles had a size of 111.7 nm, the size distribution of 35, and zeta potential of 20.8 mV (Fig. 2).

The morphological characteristics of freeze-dried chitosan nanoparticles were determined using the SEM electron microscope and it was found that the prepared chitosan nanoparticles had an orderly and homogeneous spherical with smooth edges (Fig. 3).

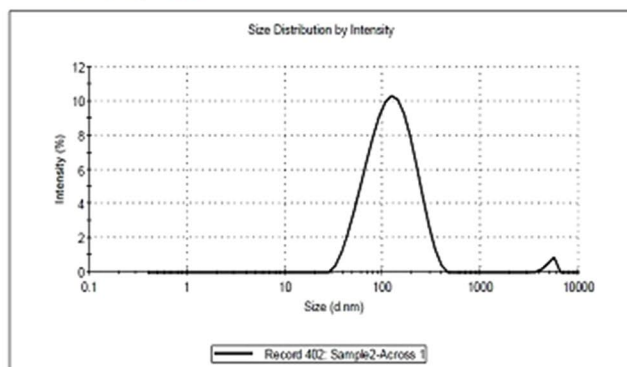
### Breast tumor formation and histological examination of tumor tissues

In this study, the treatments of BALB/c mice by DMBA and MNU chemical compounds were used for induction of breast cancer. After DMBA gavage and MNU infusion, in the following period, the visceral and breast cancer tumors

#### Results

	Diam. (nm)	% Intensity	Width (nm)
Z-Average (d.nm): 111.7	Peak 1: 134.3	98.4	68.84
Pdl: 0.241	Peak 2: 5137	1.6	518.2
Intercept: 0.696	Peak 3: 0.000	0.0	0.000

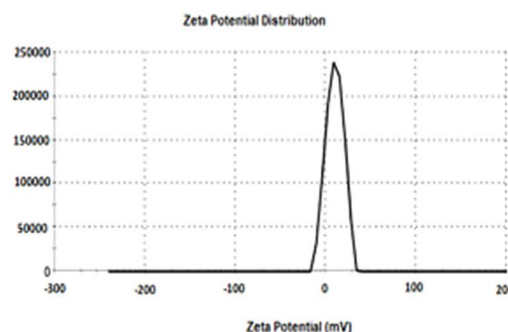
Result quality **Good**



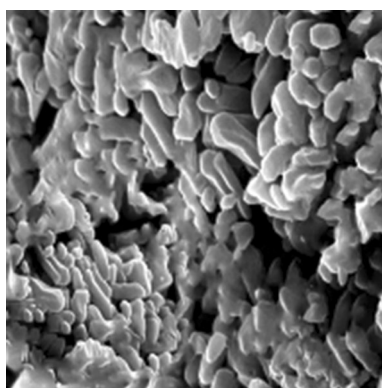
#### Results

	Mean (mV)	Area (%)	Width (mV)
Zeta Potential (mV): 20.8	Peak 1: 20.8	100.0	10.4
Zeta Deviation (mV): 10.4	Peak 2: 0.000	0.0	0.000
Conductivity (mS/cm): 2.69	Peak 3: 0.000	0.0	0.000

Result quality: **Good**



**Fig. 2** The particle size (left) and zeta potential distribution (right) of chitosan nanoparticles



**Fig. 3** The SEM image of prepared chitosan nanoparticles

**Table 3** The tumor incidence and average of breast tumor weight in the cancerous BALB/c mice in the groups of prevention and therapy categories compared to the healthy group

Group	Number of cancerous mice/number of mice	Tumor incidence (%)	Mean breast tumor weight in cancerous mice (mg)
Prevention category			
A	2/11 <sup>a,*</sup>	18.2	122 ± 6*
B	1/11 <sup>a,*</sup>	9.1	106 ± 2*
C	11/12	91.7	657 ± 4
Therapy category			
D	3/12*	25	345 ± 3*
E	2/11 <sup>a,*</sup>	18.2	268 ± 5*
F	10/11 <sup>a</sup>	90.9	723 ± 7
Healthy mice			
G	0/6	0	0

\*These data compared with C and F groups were statistically significant ( $p < 0.05$ ) using one-way ANOVA analysis

<sup>a</sup>One mouse in these groups was excluded from the study for some unknown reasons (< 10%)

were observed in some animals of groups (especially in the control group mice (C and F) and except healthy mice without any treatments). The breast tumors' average weight and tumor incidence rate in mice of each group are recorded in

Table 3. The mice that died due to the inappropriate infusion or other agents were excluded from the study (not more than 10% of each group). The visceral and breast cancer tumors in cancerous BALB/c mice are shown in Fig. 4. The rate of breast tumor incidence and the mean mammary tumor weight in mice of the therapy category were slightly larger than the prevention category. The histological study of normal (healthy mice) and breast tumor tissues were investigated by H&E staining. In some mice, after treatment of chemical compounds only inflammation of the breast tissue was observed (Fig. 5). In this study, as we expected, the breast tumor incidence in the prevention and therapy groups that received the recombinant vector was very low (according to Table 3) and breast tissues in these categories were similar to the breast tissue of healthy BALB/c mice (Fig. 5). The H&E results confirmed the creation of breast cancer tumors that were induced by chemical compounds, especially in animals of C and F groups (not received pBudCE4.1-azurin-MAM-A recombinant vector).

### Qualitative RT-PCR results

After tissue separation from the injection sites of all animal groups, RNA extraction and cDNA synthesis were done. Then, the qualitative RT-PCR was used to confirm the expression of *azurin* and *MAM-A* target genes of pBudCE4.1-azurin-MAM-A recombinant plasmid in the quadriceps muscle of animals. The RT-PCR products on 1% agarose gel electrophoresis revealed the DNA bands with the length size of 292 and 221 bp for *azurin* and *MAM-A* genes, respectively. In the animals of both prevention and therapy categories (that were infused by the pBudCE4.1-azurin-MAM-A recombinant vector alone or combined with the chitosan nanoparticles), the expressions of both target genes were observed in the tissue infusion site of the animals (Fig. 6).

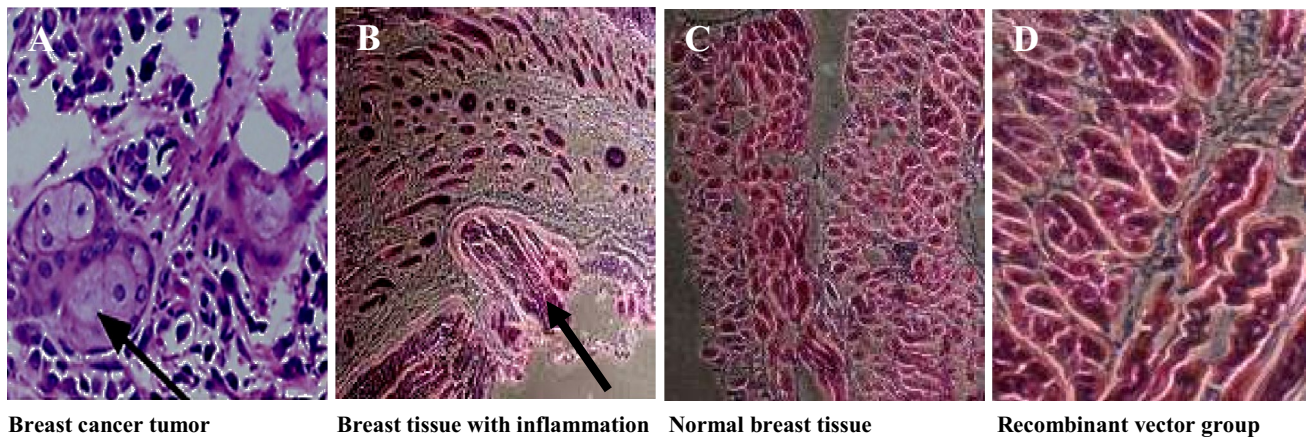
### Serological findings

The immune stimulation was evaluated in the infused BALB/c mice by pBudCE4.1-azurin-MAM-A recombinant vector alone or combined with chitosan nanoparticles by

**Fig. 4** The creation of the visceral and breast cancer tumors in treated BALB/c mice by DMBA and MNU chemical compounds

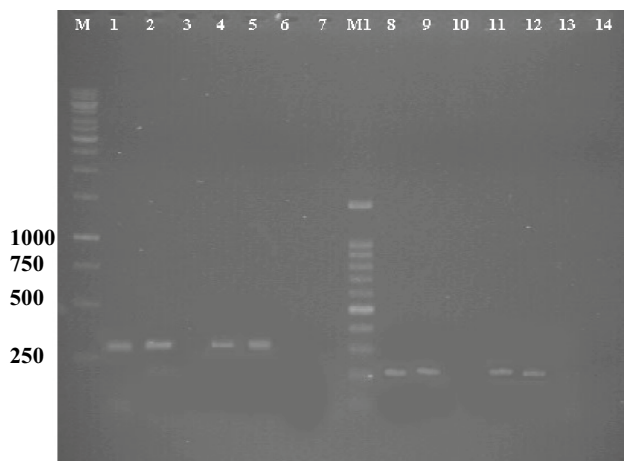






**Fig. 5** The images of H&E staining of breast tissues ( $\times 200$  magnifying). **a** Tissue of breast cancer tumor, **b** breast tissue with only inflammation, **c** normal breast tissue (healthy mouse), and **d** breast

tissue of infused mouse by pBudCE4.1-azurin-MAM-A recombinant vector without any tissue lesion



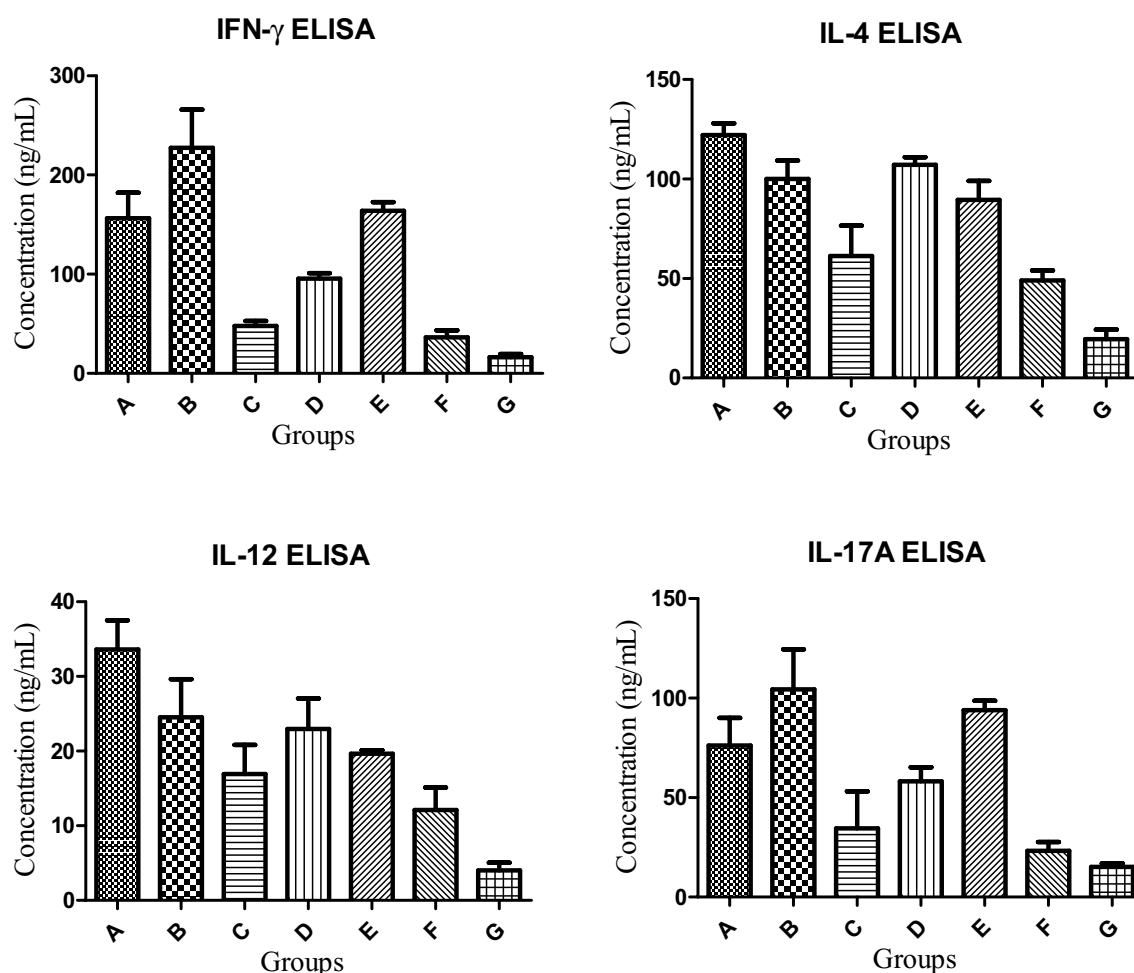
**Fig. 6** A 1% agarose gel electrophoresis for investigating the expression of *azurin* and *MAM-A* genes in infused muscles of BALB/c mice by pBudCE4.1-azurin-MAM-A recombinant vector alone and combined with nanoparticle in both prevention and therapy categories (lane M and M1 are 1-kb and 100 bp gene rulers (Thermo Fisher Scientific), respectively; lanes 1 and 2, 4, and 5: the amplified *azurin* gene in prevention and therapy classes, respectively; lane 3: the infused mouse by empty pBudCE4.1 plasmid (control group); lane 6: healthy mouse without infusion; and lane 7: negative control (non-template DNA); lanes 8, 9, 11, 12: the bands of amplified *MAM-A* gene in muscle of infused mice; lane 10: infused mouse by entire pBudCE4.1 vector; lane 13: non-infused mouse; and lane 14: negative control (without DNA), respectively)

ELISA assay. The serum levels of IFN- $\gamma$ , IL-4, IL-12, and IL-17A cytokines in collected serum specimens from all mice of each prevention and therapy classes were investigated by each specific ELISA kits. The comparisons of serum levels of cytokines by ELISA test showed that IFN- $\gamma$ , IL-12, and IL-17A serum levels in the infused mice by pBudCE4.1-azurin-MAM-A plus chitosan nanoparticles

(B and E groups) compared to the infused mice with only recombinant vector (A and D groups) in both prevention and therapy categories increased statistically significantly ( $p < 0.05$ ). In addition, the IL-4 serum range in A and B groups in prevention class compared to D and E groups in the therapy category increased, but the difference was not statistically significant ( $p > 0.05$ ). In addition, the serum levels of these cytokines in the infused mice by only recombinant vector (A and D groups) and recombinant plasmid together with nanoparticles (B and E) compared with infused mice by empty pBudCE4.1 vector (C and F groups) and healthy mice (G group without any infusions) in both prevention and therapy categories were significantly increased ( $p < 0.05$ ) (Fig. 7).

### The quantitative real-time PCR analysis

The comparisons of the relative expression of cytokine genes (*IFN- $\gamma$* , *IL-4*, *IL-12*, and *IL-17A* genes) and tumor markers (*CEA*, *Krt20*, and *Muc1* genes) after normalization via the reference gene (*GAPDH*) were done according to the Livak method. According to the graphs of Fig. 8, the expression of all cytokine genes including *IFN- $\gamma$* , *IL-4*, *IL-12*, and *IL-17A* in the mice of both prevention and therapy categories which were infused by recombinant vector alone or combined with chitosan nanoparticles (A, B, D, and E groups) compared with the infused group by empty pBudCE4.1 vector (C and F groups) and as well as non-infused mice (healthy mice; G group) elicited and significantly increased ( $p < 0.05$ ). In addition, the results showed that the expression level of cytokine genes in injected mice with the recombinant vector first and then treated with chemical compounds (A and B as a prevention category) compared to the animals of the therapy category that were first treated with cancerous



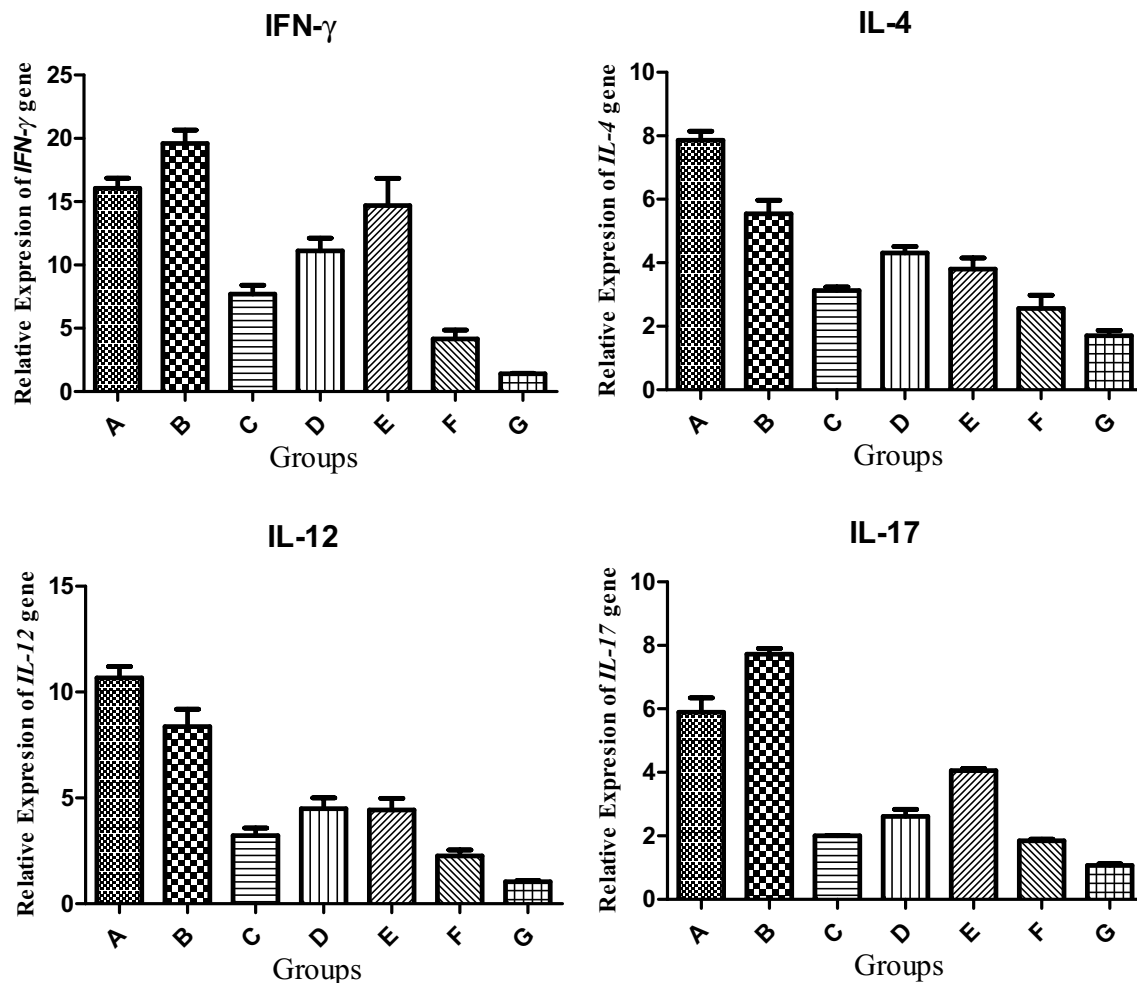
**Fig. 7** The comparisons of serum levels of IFN- $\gamma$ , IL-4, IL-12, and IL-17A cytokines in infused mice of both prevention and therapy categories (infused mice with only pBudCE4.1-azurin-MAM-A recombinant vector (A and D groups), recombinant vector plus chitosan

nanoparticles (B and E groups), empty pBudCE4.1 DNA plasmid as a control (C and F groups), and without infusion as a normal mice (G group)

chemicals and then received the recombinant plasmid (D and E groups) increased ( $p < 0.05$ ). The expression of all cytokine genes except *IL-4* and *IL-12* in infused BALB/c mice by pBudCE4.1-azurin-MAM-A recombinant vector combined with chitosan nanoparticles (B group) compared with the infused group by an only pBudCE4.1-azurin-MAM-A recombinant vector (A group) increased but is not statistically significant ( $p > 0.05$ ).

To verify the efficiency of the infused recombinant vector, the expression of mice breast tumor markers (*CEA*, *Krt20*, and *Muc1* genes) were evaluated. The results indicated that *CEA* and *Muc1* genes in the infused mice by pBudCE4.1-azurin-MAM-A recombinant vector alone and along with chitosan nanoparticles (A, B, D, and E groups) compared with injected mice with intact vector (C and F groups) infused by the only empty pBudCE4.1 plasmid) in both prevention and therapy categories significantly decreased ( $p < 0.05$ ) and represent that the development and spread of

tumors were inhibited. While, the expression diminution of *Krt20* was observed in the mice that only received recombinant vector plus chitosan nanoparticles. Overall, the significant down-regulation of *CEA*, *Krt20*, and *Muc1* tumor markers in the infused mice by recombinant vector plus chitosan nanoparticles (B and E groups) compared with other groups was most seen ( $p < 0.05$ ). In addition, in the healthy mice (G group without any treatment), a small amount of tumor markers expressions as compared to the treated mice with carcinogenic chemicals were observed. In addition, in cancerous tissues of BALB/c mice in C and F groups that were infused by pBudCE4.1 empty vector (lacking of *azurin* and *MAM-A* target genes), a considerable expression of tumor markers compared to the treated mice with a recombinant vector alone or combined with nanoparticles was observed ( $p < 0.05$ ) (Fig. 9).



**Fig. 8** The relative expression comparisons of cytokine genes in infused BALB/c mice of both prevention and therapy categories. A and D: infused mice by the only pBudCE4.1-azurin-MAM-A recombinant vector. B and E: infused mice by pBudCE4.1-azurin-MAM-

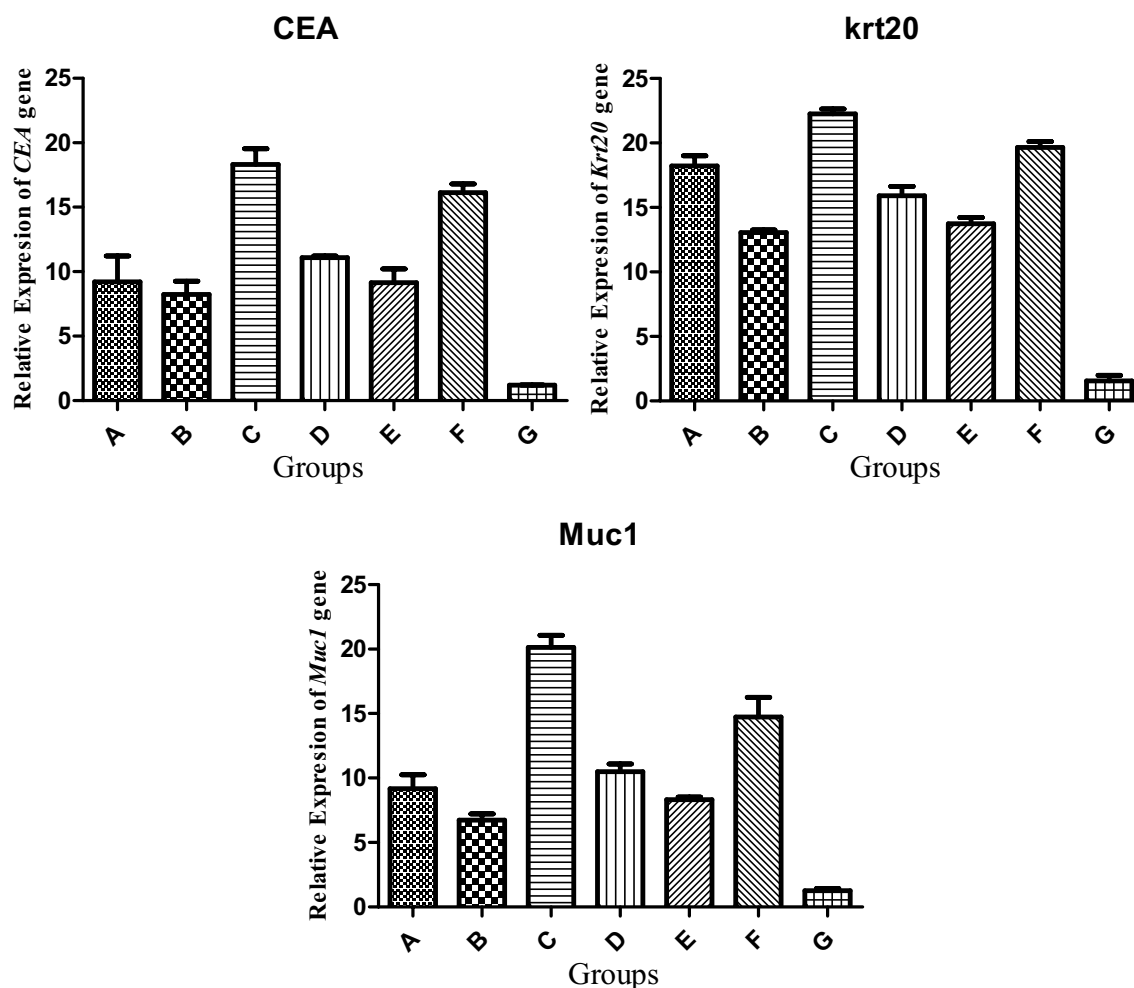
A recombinant vector plus chitosan nanoparticles. C and F: infused mice by an empty pBudCE4.1 vector. G: non-infused mice (healthy group)

## Discussion

In this study, the synchronous effects of *azurin* gene of *P. aeruginosa* and human *MAM-A* gene were evaluated in the stimulation of immune responses against breast cancer tumors. For this purpose, the pBudCE4.1-azurin-MAM-A gene construct was used for infusion of cancerous BALB/c mice that were subjected as prevention and therapy categories. As regards, the sequence of human *MAM-A* is similar to the mouse *MAM-A* antigen, we used this gene in the pBudCE4.1-azurin-MAM-A construct for induction of the immune system against breast cancer in BALB/c mice. Furthermore, the ultimate goal in the future studies wills application of this recombinant vector as a DNA vaccine in human.

In infused animals with pBudCE4.1-azurin-MAM-A recombinant plasmid in both prevention and therapy

categories (A, B, D, and E groups) compared to the injected mice by an empty pBudCE4.1 vector (C and F groups), the breast tumor incidence was decreased statistically significantly ( $p < 0.05$ ). Moreover, the histological studies using H&E staining confirmed these results. The tumor incidence and weight in animals of prevention category were slightly less than the therapy category and it was shown that in therapy group after generation of breast tumors, the pBudCE4.1-azurin-MAM-A recombinant vector decreased the diameter of the tumors. Therefore, these cancerous mice could be an appropriate animal model for investigation of the carcinogenesis effects of chemical compounds on human breast tumors and application of DNA vaccines. The successful expression of *azurin* and *MAM-A* genes in the tissues of infusion sites of BALB/c mice were observed. The ELISA test demonstrated that the serum level of IFN- $\gamma$ , IL-12, and IL-17A cytokines in



**Fig. 9** The tumor marker gene expression in BALB/c mice of prevention and therapy categories. A and D: injected mice by an only recombinant vector. B and E: injected mice by recombinant vector

plus nanoparticles. C and F: injected mice by the pBudCE4.1 (empty vector). G: non-injected mice (healthy group)

the infused mice by pBudCE4.1-azurin-MAM-A plus chitosan nanoparticles (B and E groups) compared to injected animals with only recombinant vector (A and D groups) was increased statistically significantly ( $p < 0.05$ ). These findings displayed that chitosan nanoparticles via helping to better uptake of plasmid DNA can improve the stimulation of the immune system. In addition, the ELISA test manifested that the recombinant vector can increase the serum level of these cytokines statistically significantly ( $p < 0.05$ ).

The findings of the real-time PCR indicated that this recombinant vector alone or combined with chitosan nanoparticles (A, B, D, and E groups) stimulated the immune system via increasing of the *IFN- $\gamma$* , *IL-4*, *IL-12*, and *IL-17A* cytokine gene expression. However, in infused mice by recombinant vector plus nanoparticle, the low difference in the rise of the expression level of these cytokine genes (except *IL-4* and *IL-12* interleukins) was observed ( $p > 0.05$ ).

The statistically significantly increase of these cytokines in the recombinant vector recipient mice observed by molecular test confirmed the findings of the ELISA assay on incitement of the animals' immune system. The role of *IL-12* in breast cancer is tumor suppression and has antitumor activity (Tugues et al. 2015). Thus, *IFN- $\gamma$*  secretes at the tumor site by NK cells and NKT can induce tumor cell death (Alshaker and Matalka 2011). In addition, *IL-17A* is a cytokine produced by Th17 cells and it is a proinflammatory cytokine in the breast tumor environment and correlate with poor prognosis (Cochaud et al. 2013; Esquivel-Velázquez et al. 2015). *IL-4* is a pleiotropic cytokine produced by T lymphocytes, which acts as a potent antitumor activity against various tumors (Nagai and Toi 2000; Borj et al. 2017). Therefore, the over-expression of these cytokines in the infused animals by recombinant vector that were observed in this study confirms the correct function of this gene construct against breast tumor propagation.



The results of tumor marker gene expression in the breast tissues showed that *CEA* and *Muc1* expressions in injected animals by pBudCE4.1-azurin-MAM-A recombinant vector alone or combined with nanoparticles (A, B, D, and E groups) decreased statistically significantly ( $p < 0.05$ ). While, the *Krt20* gene expression decreased only in the infused mice with a recombinant vector plus chitosan nanoparticles (B and E groups) in both prevention and therapy states. These results indicated that in the presence of nanoparticles, the recombinant vector acts better in diminution of the tumor marker expression. But until now, the direct effects of azurin protein on tumor markers of breast cancer (*CEA*, *Muc1*, and *Krt20* genes) have not been studied. Domenici and co-workers described the specific interaction of p53 with azurin and they indicated that azurin can enter into the cancer cells and via forming a specific and stable complex with human p53 which leads to increase of tumor suppressor activity (Domenici et al. 2011).

So far, no study has been done to investigate the simultaneous effects of *azurin* gene of *P. aeruginosa* and human *MAM-A* gene as a DNA vaccine on inhibition of breast cancer in laboratory animals. To our knowledge, this is the first report of co-application of *azurin* and *MAM-A* genes as a recombinant vector for prevention and therapy of induced breast cancer in exposed BALB/c mice by DMBA and MNU chemical compounds. In a study of Yamada et al. 2002, it was shown that the bacterial redox protein azurin causes the regression of human UISO-Mel-2 tumors xenotransplanted in nude mice. They indicated that this protein may potentially be used in cancer treatment, whereas in our study, the concurrent effects of azurin and human *MAM-A* as a DNA vaccine in the excitation of the immune system of BALB/c mice against induced breast cancer tumors were evaluated. Our results indicated that breast tumors in animals that received pBudCE4.1-azurin-MAM-A recombinant plasmid alone or combined with nanoparticles inhibited and this effect increased in the mice that received the recombinant vector plus chitosan nanoparticles. In one clinical trial study by Tiriveedhi and colleagues, the safety and biological efficacy of *MAM-A* DNA vaccine against breast cancer are evaluated. After vaccination, in their study, the significant increase of the frequency of *MAM-A*-specific CD8 T cells by flow cytometry and the enhanced number of *MAM-A*-specific IFN $\gamma$ -secreting T cells by enzyme-linked immunospot (ELISpot) assay was observed. They indicated that the *MAM-A* DNA vaccine is safe and capable to elect the immune system responses, but they suggested that further study must be done to evaluate the potential of the *MAM-A* DNA vaccine for breast cancer prevention and/or therapy (Tiriveedhi et al. 2014). While, in our study, we used the *MAM-A* and *azurin* genes together as a DNA vaccine alone or combined with chitosan nanoparticles for stimulation of the immune system in breast cancerous animal for the

first time. The effectiveness of pBudCE4.1-azurin-MAM-A recombinant plasmid was evaluated by serological (ELISA) and molecular (q-real-time PCR) tests. Our findings indicated that in both prevention and therapy categories, this recombinant vector can increase the cytokine gene expression (*IFN- $\gamma$* , *IL-4*, *IL-12*, and *IL-17A*) and also by diminution of the tumor marker expression (*CEA*, *Muc1*, and *Krt20* genes) inhibit the creation and expansion of breast cancer tumors. In another study, it was found the potential effects of azurin on non-small cell lung cancer (NSCLC) cells evaluated and the anticancer effects of this bacterial protein across several cancer cell lines especially breast cancer (Bernardes et al. 2016; Chakrabarty 2014). Therefore, in the present research, we used azurin bacteriocin beside human *MAM-A* as a pBudCE4.1-azurin-MAM-A recombinant vector in two modes which included prevention and therapy of breast cancer tumors in induced cancerous BALB/c mice, and the positive effects of these target genes on the stimulation of the immune system and inhibition of cancer tumors were observed.

## Conclusions

The pBudCE4.1-azurin-MAM-A recombinant vector which was generated and used in this study, via *azurin* and *MAM-A* genes delivery in BALB/c mice, has a potential ability to stimulate the immune system by increasing the cytokine gene expression and can inhibit the tumor marker expression in breast cancer tumors. It is also suggested that further studies are required to evaluate the effects and application of this recombinant vector as a DNA vaccine on breast cancer prevention and/or therapy in animal and human models.

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## Compliance with ethical standards

**Conflict of interest** The authors declare there are no conflicts of interest in this study.

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